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Nitric oxide formation and plasma L-arginine levels in pulmonary hypertensive rats

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Summary The elevation of plasma L-arginine levels stimulates nitric oxide (NO) synthesis, but the underlying mechanisms are not yet understood. We examined the role of physiological changes in pulmonary arteries on endogenous NO production. Male Wistar rats were divided into following groups: (1) control rats receiving normal water orally, (2) ARG rats receiving L-arginine water orally, (3) MCT rats injected with monocrotaline (MCT) on day 0 and receiving normal water orally, and (4) MCT + ARG rats injected with MCT on day 0 and receiving L-arginine water orally. The rats were studied after 23 days of dietary intervention. In MCT + ARG rats, supplemental L-arginine exhibited a significant pulmonary vasodilatory effect, as shown by a decreased pulmonary arterial pressure (PAP) ($P<0.001$), decreased right ventricular hypertrophy ($P<0.01$), and improved endothelium-dependent relaxation ($P<0.01$). Also L-arginine inhibited the elevation of plasma endothelin-1 ($P<0.01$). Oral L-arginine administration increased plasma L-arginine levels about twofold, but in only MCT + ARG rats (i.e., not in ARG rats) did the urinary nitrate excretion significantly increase ($P<0.05$), which is an indicator of endogenous NO formation. Oral administration of L-arginine is effective against pulmonary vascular remodeling. The data also suggest that the initial elevation of PAP is important for the induction of endogenous NO synthesis.

Introduction

The severity of pulmonary hypertension is one of the most important determinants of the prognosis of congenital and acquired heart diseases. Reduced production of nitric oxide (NO; a potent endogenous vasodilator) in endothelial cells of the pulmonary arteries contributes to the progression of pulmonary hypertension.[1] Therefore, the promotion of endogenous NO production in endothelial cells of the pulmonary arteries can be an alternative therapy for controlling pulmonary hypertension.[2,3] NO is synthesized in vascular endothelial cells by the action of endothelial NO synthase (eNOS) on L-arginine, which is converted to L-citrulline and NO,[4] and its production can be further stimulated by a variety of

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receptor agonists as well as by the shear stress produced by vascular flow.[5–7]

It has been reported that oral L-arginine administration to cholesterol-fed rabbits with atherosclerosis improves endothelium-dependent relaxation, inhibits platelet aggregation, and reduces atheroma and intimal hyperplasia.[8] Oral supplementation of L-arginine is simple to implement clinically, but there are few studies on the effects of oral administration of L-arginine in pulmonary hypertensive animal models. The elevation of plasma L-arginine levels stimulates NO synthesis, but the relationship between changes in plasma L-arginine levels and the rate of endogenous NO formation—and the underlying mechanisms—are not fully understood. We hypothesized that hemodynamic changes such as elevation of arterial pressure and/or sheer stress in pulmonary arteries might strongly affect endogenous lung NO production.

Here we describe the effects of oral administration of L-arginine on monocrotaline (MCT)-induced pulmonary hypertension in rats. We also examined the effects of physiological changes in pulmonary arteries on endogenous NO production.

Methods

Animals

The experimental procedures are shown in Fig. 1. Four-week-old male Wistar rats weighing 94–108 g (SLC, Shizuoka, Japan) were divided into four groups as follows: (i) rats injected with 0.9% saline vehicle and receiving normal water throughout the course of the study (control rats, $n=10$), (ii) rats injected with vehicle and receiving 2.25% L-arginine in their drinking water (ARG rats, $n=6$), (iii) rats injected with MCT (40 mg/kg) and receiving normal water (MCT rats, $n=21$), and (iv) rats injected with MCT (40 mg/kg) and receiving 2.25% L-arginine water (MCT + ARG rats, $n=22$). This oral dosage of L-arginine water has been shown to restore NO production in hypercholesterolemic animals.[9] MCT (Aldrich, WI, USA) was dissolved in 1.5 ml of 1 N HCl, adjusted to pH 7.4 with 1 N NaOH, and diluted with distilled water to a concentration of 20 mg/ml. The rats were given a single subcutaneous injection of either MCT or vehicle on day 0, and then studied after 23 days of dietary intervention. They were given food and water ad libitum and housed in 12 h/12 h light/dark conditions at $23\pm0.5^{\circ}$C and a relative humidity of 55 ± 5%. All experiments were performed according to protocols approved by the animal use and care committee of Nagoya City University Graduate School of Medical Sciences.

Collection and measurement of urine excretion of nitrate

On day 22 after the injection of MCT, the rats were placed in metabolic cages for 24 hours to collect urine samples. Each sample was frozen at -20°C until the urine concentration of nitrate, a metabolite of L-arginine in NO production, was measured by high-performance liquid chromatography (HPLC; SRL, Tokyo, Japan).

Measurement of systemic arterial pressure and pulmonary arterial pressure

After completing the collection of urine samples (on day 23), the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (33 mg/kg). An arterial catheter of silastic tube (0.31 mm ID and 0.64 mm OD) flushed with heparinized saline was inserted via the left carotid artery into the descending aorta, and another catheter was inserted via the right jugular vein into the pulmonary artery by a closed-chest technique under spontaneous breathing.[10] Both systemic arterial pressure and pulmonary arterial pressure (PAP) were measured using a pressure transducer and amplifier system (IB-160, Fukuda Denshi, Tokyo, Japan).

Blood sampling and measurement of plasma L-arginine and endothelin-1 levels

Blood samples were drawn from the left carotid artery and collected into plastic tubes containing heparin for the measurement of L-arginine, and containing EDTA (ethylenediaminetetraacetic acid) and aprotinin for the measurement of endothelin-1 (ET-1). The samples were then centrifuged at 2000*g* for 10 min at 4°C to isolate the plasma. The plasma was frozen at −20°C until its concentrations of L-arginine and ET-1 were measured.

The plasma concentration of L-arginine was measured by HPLC. The plasma concentration of ET-1 was measured using a commercially available ET-1,21-specific radioimmunoassay system (SRL).

Examination of ventricular weight

The heart and lungs were removed and weighed, and then the right ventricular free wall was separated from the left ventricle and septum. The organ-to-body-weight ratios were calculated for the respec-

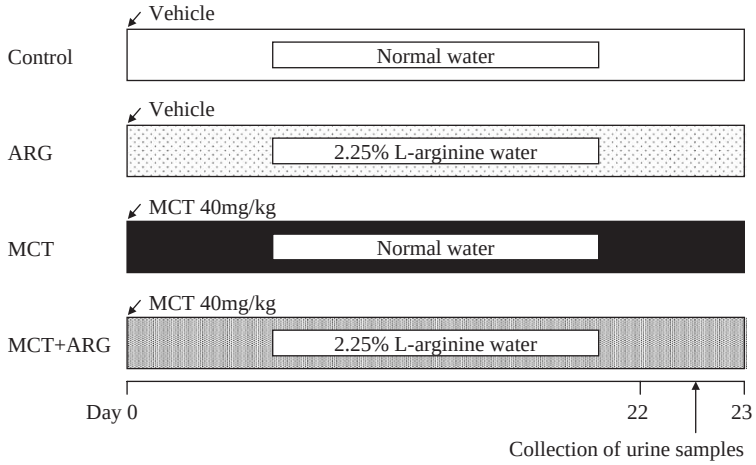

Figure 1 Experimental procedures.

tive organs. Specifically, we used the ratio of the weight of the right ventricle to the weight of the left ventricle plus septum [RV/(LV + S)] as an index of right ventricular hypertrophy.

Measurement of endothelium-dependent relaxation of the pulmonary artery

Pulmonary arteries were separated carefully from both the heart and lungs and placed in modified Krebs–Henseleit solution at room temperature in preparation for measuring the endothelium-dependent relaxation. The composition of the solution was as follows (in mM): Na^+, 134; K^+, 5.9; Mg^{2+}, 1.2; Ca^{2+}, 2.5; HCO_3^-, 15.5; $H_2PO_4^-$, 1.2; Cl^-, 137; and glucose, 15.5. A segment of the intrapulmonary artery was dissected and cut. A 2-mm-long ring segment of the intrapulmonary artery was cleaned of fat and connective tissue and suspended vertically between two stainless steel wires in a recording chamber containing the buffer solution. One of the wires was anchored and the other was connected to a mechanoelectric transducer (TB-612 T, Nihon Kohden, Tokyo, Japan) connected to a carrier amplifier (SEN-6102 M, Nihon Kohden, Tokyo, Japan) to measure isometric forces.

Cumulative concentration–response relaxation curves to acetylcholine were obtained in each ring. After a steady state of precontraction with noradrenaline (10^{-6} M) was reached, the dose–response characteristic to acetylcholine (10^{-8}–10^{-5} M) was obtained to assess the endothelial vasodilatory response. (The relaxation responses presented here are expressed as a percentage of the precontractile tension induced with noradrenaline.) The rings were allowed to re-equilibrate in drug-free buffer for 40 min until the tension between experimental cycles returned to the original baseline. The responses were recorded using a chart recorder (VP-6524A, Panasonic, Tokyo, Japan).

Statistical analysis

All the data are expressed as means ± SE. For hemodynamic assessment, measurement of right ventricular weight, and analysis of plasma L-arginine levels, ET-1 levels, and urinary nitrate excretion, data were analyzed statistically using one-way ANOVA and followed by Scheffe's *F* test when significant differences were found. Changes in body weight and endothelium-dependent relaxation responses were analyzed with repeated-measures ANOVA followed by Scheffe's *F* test, and comparisons among the groups at each measuring points were made by one-way ANOVA followed by Scheffe's *F* test. Differences with a *P* value less than 0.05 were considered significant.

Results

Body weight

The changes in body weight are summarized in Table 1. All rats gained weight steadily, but compared with control and ARG rats, body-weight gains in MCT and MCT + ARG rats were inhibited from day 7 to day 23 ($P < 0.001$). Treatment with L-arginine did not affect body-weight gain.

Progressive changes in hemodynamics, right ventricular hypertrophy, and plasma ET-1 levels

Mean PAPs are shown in Fig. 2A. MCT rats exhibited significant pulmonary hypertension, as indicated by

the PAP (41.6 ± 1.4 mmHg) being higher than that in control rats (21.4 ± 0.6 mmHg, $P<0.001$). Treatment with L-arginine in MCT + ARG rats reduced the elevation of PAP (30.4 ± 1.4 mmHg, $P<0.001$ vs. MCT rats). However, L-arginine treatment did not affect PAP in ARG rats (20.7 ± 0.6 mmHg). Treatment with L-arginine did not produce any significant differences in the PAPs of normal rats.

The systemic arterial pressure was the same in all groups: 113.2 ± 4.1 mmHg in control rats, 115.5 ± 4.2 mmHg in ARG rats, 115.6 ± 4.5 mmHg in MCT rats, and 112.2 ± 3.5 mmHg in MCT + ARG rats (Fig. 2B). Treatment with L-arginine did not influence systemic arterial pressure.

MCT rats developed right ventricular hypertrophy, and RV/(LV + S) was higher in MCT rats (0.44 ± 0.03) than in control rats (0.25 ± 0.01, $P<0.001$; Fig. 2C). Treatment with L-arginine attenuated right ventricular hypertrophy, as shown by the decrease in RV/(LV + S) in MCT + ARG rats (0.34 ± 0.02, $P<0.01$ vs. MCT rats). There were no significant differences in RV/(LV + S) between control rats and ARG rats (0.24 ± 0.01).

The plasma ET-1 concentration in MCT rats (9.2 ± 1.0 pg/ml) was higher than in control rats (4.3 ± 0.6 pg/ml, $P<0.001$; Fig. 2D). Treatment with L-arginine in MCT + ARG rats markedly inhibited this elevation of plasma ET-1 levels (5.3 ± 0.3 pg/ml, $P<0.01$ vs. MCT rats). There were no statistically significant differences in plasma ET-1 levels among control, ARG (4.5 ± 1.0 pg/ml), and MCT + ARG rats.

Plasma L-arginine levels and urinary excretion of nitrate

Plasma L-arginine concentrations increased significantly in both ARG and MCT + ARG rats (420 ± 30 nmol/ml and 322 ± 53 nmol/ml, respectively) compared to that in control rats (158 ± 10 nmol/ml, $P<0.001$ and $P<0.01$, respectively; Fig. 3A). In MCT rats, the plasma concentration of L-arginine was slightly reduced (115 ± 16 nmol/ml), but the difference was not significant.

Urine excretion of nitrate increased after 23 days of oral administration of L-arginine in MCT + ARG rats (29.9 ± 1.7 nmol/g BW/day) compared to that in control rats (19.6 ± 2.3 nmol/g BW/day, $P<0.05$; Fig. 3B). However, the level of urine nitrate in ARG rats was not elevated (19.3 ± 3.9 nmol/g BW/day). The urine nitrate level in MCT rats was slightly reduced (17.4 ± 2.0 nmol/g BW/day), but the difference was not significant.

Effect of long-term oral administration of L-arginine on endothelium-dependent relaxation

Figure 4 shows that the endothelium-dependent relaxation response to acetylcholine (10^{-8}–10^{-5} M) in MCT rats was lower than that in control rats ($P<0.001$). The relaxation response in MCT + ARG rats was also less than that in control rats ($P<0.001$), but it was greater than that in MCT rats ($P<0.01$). The MCT injection damaged endothelium-dependent vasodilation, but this negative effect was inhibited by oral administration of L-arginine.

Discussion

NO is very rapidly oxidized to nitrite and nitrate in vivo, and the greater part of the metabolites is excreted into the urine in the form of nitrate, so that urinary nitrate excretion has been suggested to be a reliable indicator of NO formation.[11,12] In the present study, oral administration of L-arginine had a significant pulmonary vasodilatory effect and prevented vascular endothelium dysfunction. Moreover, the pulmonary vasodilatory effect and the

Table 1 Body-weight changes after MCT or saline injection.

		Body weight (g)				
		Day 0	Day 7	Day 14	Day 21	Day 23
Control	($n=8$)	101 ± 1	146 ± 1	199 ± 2	247 ± 3	256 ± 3
ARG	($n=6$)	101 ± 1	138 ± 4	207 ± 4	250 ± 4	264 ± 5
MCT	($n=21$)	98 ± 1	126 ± 2***	172 ± 2***	213 ± 3***	214 ± 3***
MCT + ARG	($n=20$)	99 ± 1	129 ± 2***	176 ± 2***	218 ± 4***	220 ± 4***

Effects of MCT injection and oral L-arginine administration on body-weight gain in rats. Final body weights were lower in MCT and MCT + ARG rats than in control and ARG rats. Treatment with L-arginine did not affect body-weight gains.
***$P<0.001$ vs. control rats at the same time points.

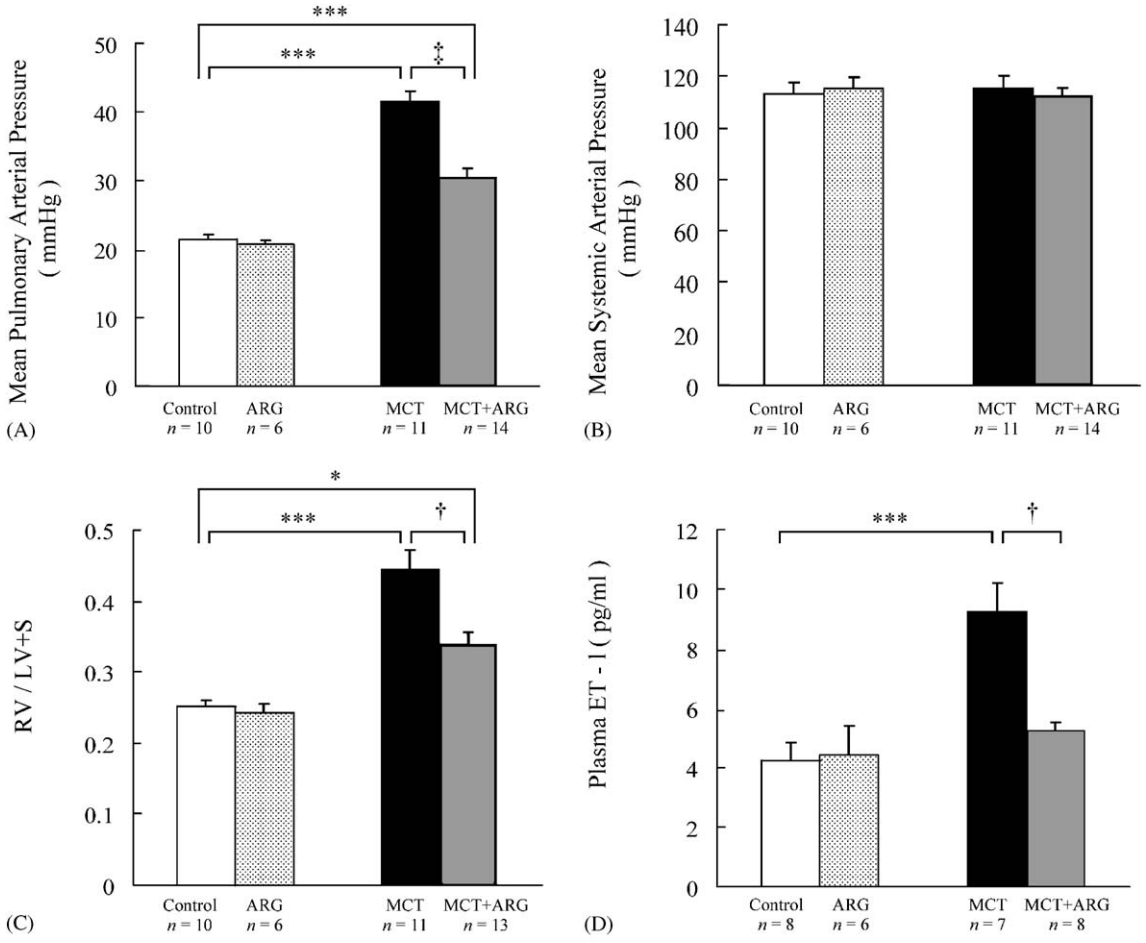

Figure 2 Effects of oral L-arginine administration for 23 days on mean PAP, mean systemic arterial pressure, RV/(LV + S), and plasma ET-1 levels in rats injected with either MCT or vehicle. L-Arginine significantly reduced mean PAP and attenuated right ventricular hypertrophy in MCT + ARG rats, but not in ARG rats. Treatment with L-arginine markedly inhibited the elevation of plasma ET-1 levels. *$P<0.05$ vs. control rats, ***$P<0.001$ vs. control rats, †$P<0.01$ vs. MCT rats, ‡$P<0.001$ vs. MCT rats.

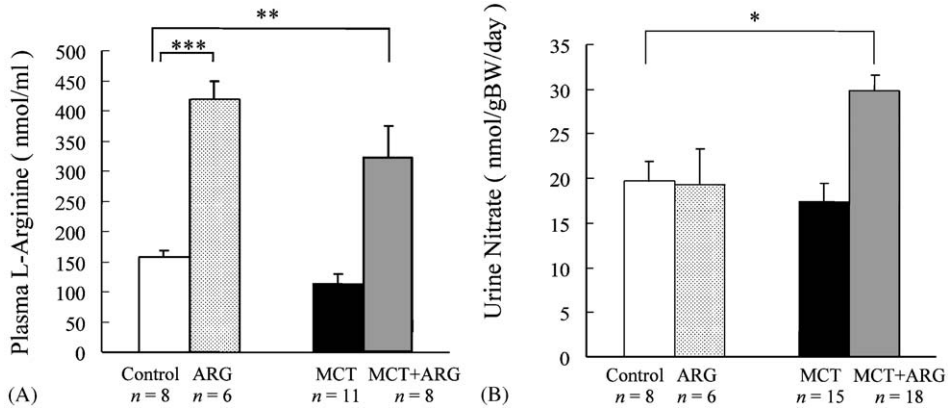

Figure 3 Oral L-arginine administration increased plasma L-arginine levels, but urine nitrate levels increased only in MCT + ARG rats. *$P<0.05$ vs. control rats, **$P<0.01$ vs. control rats, ***$P<0.001$ vs. control rats.

elevation in urinary nitrate excretion occurred selectively in rats treated with MCT and receiving L-arginine orally. NO is derived from many sources besides endothelial cells in pulmonary arteries such as in systemic arteries or kidneys.[6,13] Although there is the possibility that increased urine nitrate

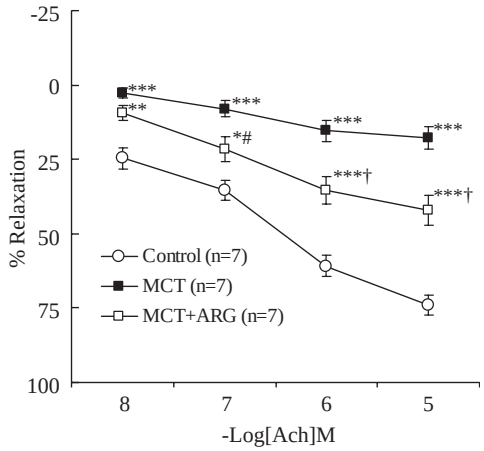

Figure 4 Effects of long-term oral administration of L-arginine on the endothelium-dependent relaxation response to acetylcholine (10^{-8}–10^{-5} M). Precontraction induced by noradrenaline (10^{-6} M) was taken as 100%. *$P<0.05$ vs. control rats at the same dose of acetylcholine, **$P<0.01$ vs. control rats at the same dose of acetylcholine, ***$P<0.001$ vs. control rats at the same dose of acetylcholine, ♯$P<0.05$ vs. MCT rats at the same dose of acetylcholine, †$P<0.01$ vs. MCT rats at the same dose of acetylcholine.

reflects NO synthesis in other tissues except lungs, the specific pulmonary vasodilatory effect and prevention of vascular endothelium dysfunction in MCT rats with L-arginine suggest that lung NO production is the most important and contributes to the amelioration of pulmonary hypertension.

In our studies, urine excretion of nitrate did not increase in normal rats receiving L-arginine, but only increased in rats treated with MCT and receiving L-arginine orally. Also in hemodynamic studies, supplementation with L-arginine did not affect PAP and systemic arterial pressure in normal rats. We speculate that administration of L-arginine had no effect on urine excretion and hemodynamics in normal rats because they were not subjected to an initial elevation of PAP and other hemodynamic forces sufficient to induce endogenous NO production. This finding is consistent with the idea that the concentration of endogenous L-arginine in endothelial cells in normal pulmonary vascular beds (as in other fresh vascular tissues) is sufficient to prevent rate limiting of the first step in the conversion of L-arginine to NO.[14] A single subcutaneous injection of MCT produces vascular endothelial damage, medial-wall thickening, and a progressive decrease in lumen diameter in pulmonary arteries.[15,16] These effects will increase the fluid sheer stress in the pulmonary arteries when blood flow does not change. Previous in vitro experiments showed that the release of NO, as assessed by measuring increases in soluble guanylate cyclase activity in the presence of acetylcholine, was stimulated by high shear stress.[5] Other experimental data indicate that pulmonary eNOS expression in vivo is upregulated by shear stress and increased pulmonary blood flow.[7] Vascular endothelial cells respond to mechanical stress and hemodynamic forces by altered gene expression and by undergoing significant structural reorganization.[17] Taken together with our results, it is conceivable that a physiological stimulus in vivo, such as a change in PAP, increases of fluid shear stress or pulsatile stretching of the vessel wall, is necessary to induce a vasodilatory effect via the L-arginine–NO pathway.

It has been reported that eNOS plays major roles in NO induction in terms of the vasodilatory effect and the prevention of vascular remodeling in pulmonary hypertension.[18,19] In contrast, the role of inducible NO synthase (iNOS) is not clearly understood,[20] and there have also been discrepancies between iNOS mRNA expression and iNOS protein levels in animal models.[21,22] iNOS expression is known to be induced by lipopolysaccharide and inflammatory cytokines.[23] A recent study reported an association between iNOS expression and advanced lesion of human pulmonary plexogenic arteriopathy caused by flow-associated pulmonary hypertension.[24] In MCT models, the severe inflammatory changes in lung tissue mean that the contribution of iNOS to NO production in the lungs has to be considered. The present study did not examine the influence of lung iNOS on endogenous NO synthesis and vascular remodeling, and hence further functional studies involving various forms and severities of pulmonary hypertension are needed to clarify this issue.

Endothelium-dependent relaxation has been shown to be impaired in patients with chronic obstructive lung disease,[25] and the vasodilatory response to acetylcholine is diminished in experimental pulmonary hypertensive animal models.[26] In the present study, administration of L-arginine in drinking water for 23 days improved endothelium-dependent relaxation of isolated pulmonary artery rings in MCT-induced pulmonary hypertension. This finding suggests that in vivo administration of L-arginine is important to the efficacy of NO production and maintenance of endothelium function in the pulmonary artery.[14] Previous experiments on endothelium-dependent relaxation of pulmonary arteries showed that in vitro perfusion of L-arginine restored the endothelium-dependent vasodilatory response in perfused lung models from MCT-treated and hypoxic rats.[14,27] In contrast, incubating pulmonary artery rings isolated from either MCT-

treated and hypoxic rats in an L-arginine organ bath did not affect the endothelium-dependent vasodilatory response.[28,29] Arterial ring segments are not subjected to the same forces as pulmonary arteries in situ.[26] These discrepancies in the vasodilatory effects of L-arginine between perfused lungs and isolated pulmonary rings may therefore be attributable to a difference in the intraluminal perfusion pressure of the pulmonary arteries between the two methods.[27]

Previous studies demonstrated that tissue and plasma levels of ET-1, a potent endothelium-derived contracting factor, are elevated both in animal models and in patients with pulmonary hypertension,[30,31] with plasma ET-1 correlating particularly well with the extent of pulmonary hypertension and reflecting the abnormalities of pulmonary circulation.[32,33] The present study also demonstrates that oral L-arginine administration successfully inhibited the elevation of plasma ET-1 levels, indicating that this treatment is beneficial in view of the maintenance of pulmonary circulation.

In summary, oral administration of L-arginine is effective against vascular remodeling via an L-arginine–NO pathway in severe experimental pulmonary hypertension induced by MCT. In human studies, administration of L-arginine has a beneficial effect in patients with pulmonary hypertension.[34,35] It is possible that oral supplementation of L-arginine will be a useful clinical therapy for pulmonary hypertension, although the safety of its long-term administration still needs to be examined.

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